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Inhibitory effects of chlorogenic acids from green coffee beans and cinnamate derivatives on the activity of porcine pancreas α -amylase isozyme I

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Abstract

The nine kinds of chlorogenic acids (CGAs) account for 80% of the total CGA content in green coffee beans. They consist of three subgroups of caffeoylquinic acids (CQAs), feruolylquinic acids (FQAs), and dicaffeoylquinic acids (diCQAs). We previously reported that the inhibitory effects of 5-CQA on the porcine pancreas α -amylase (PPA) isozymes, PPA-I and PPA-II. In this paper, we investigated the PPA-I inhibition by eight kinds of CGAs. Their IC_{50} values of CQAs, FQAs, and diCQAs against the PPA-I-catalyzed hydrolysis of *p*-nitrophenyl- α -D-maltoside were 0.08–0.23 mM, 1.09–2.55 mM, and 0.02–0.03 mM, respectively. All CQAs and FQAs and 3,5-diCQA showed mixed-type inhibition with binding to the enzyme-substrate complex (ES) stronger than to the enzyme (E). 3,4-DiCQA and 4,5-diCQA showed mixed-type inhibition, but, conversely are suggested to bind to E stronger than ES.

KEYWORDS: α -amylase inhibitor; chlorogenic acids; cinnamate; coffee; enzyme inhibition

1. Introduction

The chlorogenic acids (CGAs) refer to a family of esters between quinic acid and one or plural cinnamate derivatives such as caffeic, ferulic and *p*-coumaric acids. CGAs are widely distributed in other plants such as yacon, prune, potato, and sweet potato (Islam et al., 2002; Takenaka et al., 2003). Various biological activities of CGAs have been reported such as antioxidant activity, antimutagenicity, cancer suppression, matrix metalloproteinase inhibition, tyrosinase inhibition, DNA-methylation inhibition, and so forth (Iwai et al., 2004).

Coffee is one of the most daily consumed drinks in the world, and a major source of polyphenols, in particular, chlorogenic acids (CGAs), in human diet. Green coffee beans are richly contained CGAs, and their contents are 3.5–7.5% (w/w-dry matter) for *Coffea arabica* and 7.0–14.0% (w/w-dry matter) for *Coffea canephora* (Ky et al., 2001). The nomenclature of CGAs is based on the IUPAC numbering system (1976), and 5-caffeoylquinic acid (5-CQA) is generally called chlorogenic acid. Thirty-four kinds of CGAs have been reported in green coffee beans (Clifford et al., 2006). The CGAs in green coffee beans consist of three main classes: caffeoylquinic acids (CQAs) with three isomers (3-, 4-, and 5-CQA), dicaffeoylquinic acids (diCQAs) with three isomers (3,4-, 3,5-, and 4,5-diCQA), and feruloylquinic acids (FQAs) with three isomers (3-, 4-, and 5-FQA). These nine kinds of CGAs account for 80% of the content of total CGAs in green coffee beans. During roasting, there is a progressive destruction and transformation of CGAs with some 8–10% being lost for every 1% loss of dry matter (Clifford, 1999). The color, flavor, and aroma of coffee originate from CGAs in green coffee beans by roasting. Seventy–200 mg and 70–350 mg of CGAs are contained per a cup (200-ml) of Arabica coffee and Robusta coffee, respectively.

Porcine pancreas α -amylase (EC 3.2.1.1, hereafter abbreviated as PPA) is an endo-glucanase catalyzing the hydrolysis of α -1,4-glucosidic linkages in starch, amylose, amylopectin, and glycogen. PPA is composed of 496 amino acid residues and shows 83% homology with human pancreas α -amylase (Pasero et al., 1986). Two active components were separated from crystalline preparation of

PPA in anion-exchange chromatography, and the rapidly-eluted component was designated PPA-I and the slowly-eluted one was PPA-II (Marchis-Mouren & Pasero, 1967; Sakano, Takahashi, & Kobayashi, 1983). These isozymes are secreted in about equal amounts into pancreatic juice. The amino acid sequence of PPA-I is known (Kluh, 1981), whereas only a partial sequence is reported for PPA-II (Meloun, Kluh, & Moravek, 1980). PPA-I and PPA-II have almost the same molecular mass. The optimum pH values and temperatures for both isozymes in the starch-hydrolysis activity are 6.9 and 53 °C, and their isoelectric points are 6.5 and 6.1, respectively (Sakano, Takahashi, & Kobayashi, 1983).

α -Amylase inhibitors (AAIs) seem to be effective for the prevention and therapy of metabolic syndromes such as type II diabetes and obesity in controlling the elevation of plasma blood glucose levels by delaying postprandial carbohydrate digestion and absorption. AAI from white beans (*Phaseolus vulgaris*) was reported to reduce glycemia in both nondiabetic and diabetic animals and reduced the intake of food and water (Tormo et al., 2006). AAI from chestnut astringent skin extract was reported to suppress the rise in plasma glucose level after boiled-rice loading in a dose-dependent manner in humans (Tsujita, Yakaku, & Suzuki, 2008). Various AAIs such as acarbose (Al Kazaz et al., 1998) and protein AAI from wheat kernel (Oneda, Lee, & Inouye, 2004) are discussed in the inhibition mechanism against PPA.

We previously reported that the inhibitory effect of 5-CQA, and its components, caffeic acid (CA) and quinic acid (QA), on the PPA isozymes, PPA-I and PPA-II, using *p*-nitrophenyl- α -D-maltoside (G₂-*p*NP) as substrate at pH 6.9 and 30 °C (Narita & Inouye, 2009). the effects by other CGAs contained in green coffee beans are remained to be elucidated.

In this study, based on our previous report, the inhibition effects of eight kinds of CGAs (3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA) and eight cinnamate derivatives [cinnamic acid (CiA), *m*-methoxycinnamic acid (*m*-MCiA), *p*-methoxycinnamic acid (*p*-MCiA), ferulic acid (FA), isoferulic acid (IFA), *p*-coumaric acid (*p*-CoA), *m*-coumaric acid (*m*-CoA), and dihydro caffeic acid (DHCA)] against PPA-I and the structure-function relationship were evaluated. Figure 1 shows the structures of CGAs and cinnamic acid derivatives used for this study. We describe the kinetic

and thermodynamic analysis of the inhibitory effects of 5-FQA, 3,5-diCQA, and 4,5-diCQA (as the representatives of FQAs and diCQAs in green coffee beans) and discuss their inhibitory mechanisms. We also provide some insights into the nutritional significance of the inhibitors and their preventive effects against diabetes and obesity.

(Fig. 1)

2. Materials and methods

2.1. Materials and reagents

G₂-pNP (lot B76297-1) was purchased from Calbiochem (San Diego, CA). 3,4-Dihydroxyhydrocinnamic acid [synonym: dihydrocaffeic acid (DHCA)] was from Sigma (St. Louis, MO). *m*-Hydroxycinnamic acid [*m*-coumaric acid (*m*-CoA)], 3-hydroxy-4-methoxycinnamic acid [isoferulic acid (IFA)], *p*-methoxycinnamic acid (*p*-MCiA), and 3-methoxycinnamic acid (*m*-MCiA) were from Wako Pure Chemical (Osaka, Japan). Pancreatin, chlorogenic acid hemihydrate, caffeic acid (CA), D-(-)-quinic acid (QA), trans-cinnamic acid (CiA), 4-hydroxy-3-methoxycinnamic acid [ferulic acid (FA)], trans-*p*-coumaric acid (*p*-CoA), and all other chemicals were of reagent grade and were from Nacalai Tesque (Kyoto, Japan). Decaffeinated green coffee bean extract (DGCBE) was obtained from Ominedo Pharmaceutical Industry (Nara, Japan).

2.2. HPLC analysis of nine kinds of CGAs in DGCBE

HPLC analysis of CQAs, FQAs, and diCQAs in DGCBE was performed according to the procedures previously reported (Iwai et al., 2004; Matsui et al., 2007) with some modifications. The sample solution was applied to reversed-phase column chromatography in a preparative HPLC 7400 system

(GL Science, Tokyo) on an Inertsil ODS-3 [4.6 mm (inner diameter or ID) × 15.0 cm] column (GL Science) at the column temperature of 35 °C. The mobile phase was composed of solvents A (50 mM acetic acid in H₂O) and B [50 mM acetic acid in acetonitrile], and the gradient program was as follows: 0–30.0 min, 5–20% (v/v) of B; 30.0–45.0 min, 20–35% (v/v) of B; 45.0–50.0 min, 35–80% (v/v) of B; 50.0–50.1 min, 80–5% (v/v) of B; 50.1–60 min, 5% (v/v) of B. The injection volume of the sample solution was 10 µl and a flow-rate was 1.0 ml/min. CGAs were detected by absorption at 325 nm with a photodiode array. The spectra of CQAs, FQAs, and diCQAs exhibit the absorption maximum close to 325 nm and the shoulder at 300 nm (Murata, Okada, & Homma, 1995). CGAs in DGCBE were identified by comparing the retention times and the UV spectra of nine standard materials; 5-CQA purchased from Nacalai Tesque and 8 CGAs (3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA) purified in our laboratory according to the method previously reported (Iwai et al., 2004; Matsui et al., 2007).

2.3. Purification of eight kinds of CGAs and PPA-I

3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA were purified from DGCBE according to the procedures previously reported (Iwai et al., 2004; Matsui et al., 2007) with some modifications. Two grams of DGCBE were dissolved in 5 ml of distilled water. Five milliliters of the DGCBE solution was applied to a Sephadex LH-20 column [26 mm (ID) × 45 cm] (GE Healthcare Bio-Science, Piscataway, NJ) using 17 mM acetic acid at a flow-rate of 1.0 ml/min and 25 °C. First, the non-adsorbed fractions (10 ml each) having absorption at 325 nm were collected and packed into one fraction, which was named as the NF fraction. NF was concentrated under reduced pressure at 40 °C with a rotary evaporator, freeze-dried, and stored at -20 °C. Then, the adsorbed fractions on the Sephadex LH-20 column were eluted with 70% (v/v) ethanol, and were collected by 10 ml each. The fractions having absorption at 325 nm were packed into one fraction, which was named as the AF fraction, as well. AF was concentrated, freeze-dried, and stored in the same way as applied with NF. 0.4

grams of NF was dissolved in 2 ml of distilled water. The NF sample (2 ml) was applied to size-exclusion chromatography on a Toyopearl HW-40F column [26 mm (ID) × 45cm] (Tosoh, Tokyo, Japan), and eluted with 17 mM acetic acid at a flow-rate of 0.5 ml/min and 25 °C. The fractions (3 ml each) were collected using a fraction collector. Three peaks (peaks 1-3, hereafter designated as PF-1, PF-2, and PF-3) were obtained (see Fig. 3A). Each peak fraction was concentrated under reduced pressure at 40 °C with an evaporator. 0.4 grams of AF was dissolved in 2 ml of distilled water. Two milliliters of the solution was applied to a Toyopearl HW-40F size-exclusion chromatography column (Tosoh), and successively eluted with a stepwise gradient of 25% (v/v) and 30% (v/v) ethanol containing 17 mM acetic acid at a flow-rate of 1.0 ml/min and at 25 °C (see Fig. 3B). Two peaks (peaks 4 and 5, hereafter designated as PF-4 and PF-5) were obtained. Each peak fraction was concentrated at 40 °C with an evaporator. The PFs, from PF-1 to PF-5, were applied to reversed-phase column chromatography with a preparative HPLC PLC-561 system (GL Science) on an Inertsil ODS-3 column [20 mm (ID) × 25 cm] (GL Science) at a column temperature of 40 °C. The mobile phase was composed of solvents A (20% methanol in 35 mM acetic acid) and B (methanol), and the gradient program was as follows: 0–60.0 min, 0–50% (v/v) of B; 60.0–70.0 min, 50–100% (v/v) of B; 70.0–70.1 min, 100–0% (v/v) of B; 70.1–80 min, 0% (v/v) of B. The injection volume of the sample solution was 5 ml and a flow-rate was 15.0 ml/min. CGAs were detected by absorption at 325 nm. The purified CGAs were freeze-dried and stored at -20 °C.

PPA-I was purified from pancreatin according to the procedures previously reported (Narita & Inouye, 2009).

2.5. Inhibition of PPA-I by CGAs using *G*₂-*p*NP as substrate

PPA-I hydrolyzes *G*₂-*p*NP to produce *p*-nitrophenol (*p*NP) and maltose (Oneda, Lee, Inouye, 2004). Hydrolysis of *G*₂-*p*NP by 0.8 μM PPA-I in 20 mM sodium phosphate buffer (pH 6.9, buffer B) containing 25 mM NaCl was measured continuously following the increase in absorbance at 400 nm

due to *p*NP at 20-40 °C. The amount of *p*NP was evaluated using the molar absorption coefficient of 9.47 mM⁻¹ cm⁻¹ at pH 6.9 (Oneda, Lee, & Inouye, 2004). The molecular activity (k_{cat}) and Michaelis constant (K_m) were calculated from Hanes–Woolf plots (Segal, 1975). All measurements were performed in triplicate. Relative activity (%) in the presence of inhibitor was calculated by Equation 1 using the initial velocities v_e and v_i in the absence and presence of the inhibitor, respectively

$$\text{Relative activity (\%)} = 100 \times (v_i / v_e) \quad (1)$$

The IC₅₀ value was defined as the inhibitor concentration required for giving the relative activity of 50%. The type of inhibition was determined by Hanes–Woolf plots and Lineweaver–Burk plots. The inhibitor constants (K_i and K_i') were defined using the following general equation (Equation 2) for mixed-type inhibition (Segal, 1975):

$$v_i = V_{max} [S] / \{ K_m(1 + [I]/K_i) \} + \{ [S] (1 + [I]/K_i') \} \quad (2)$$

In Equation 2, [S] and [I] are the initial concentrations of substrate and inhibitor, respectively; V_{max} is the maximum velocity observed under the condition, $[S] \gg K_m$, in the absence of inhibitor; K_i is the inhibitor constant or the dissociation constant for the enzyme-inhibitor (EI) complex into the inhibitor (I) plus enzyme (E), and K_i' is that for the enzyme-substrate-inhibitor (ESI) complex into I plus the enzyme-substrate (ES) complex.

The standard enthalpy change (ΔH°) for the binding of the PPA-I with the inhibitor (5-FQA and 4,5-diCQA) was determined from the slope ($\Delta H^\circ/R$) of the plots of natural logarithms of $1/K_i$ and $1/K_i'$ against $1/T$ (van't Hoff plots). The standard Gibbs energy change (ΔG°) and standard entropy change (ΔS°) for the binding of PPA with the inhibitor were determined according to Equations 3 and 4.

$$\Delta G^\circ = - RT \ln (1/K_i) \quad \text{or} \quad \Delta G^\circ = - RT \ln (1/K_i') \quad (3)$$

$$\Delta S^{\circ} = (\Delta H^{\circ} - \Delta G^{\circ})/T \quad (4)$$

where R is the gas constant and T is the temperature in the Kelvin unit. Hereinafter, the thermodynamic parameters calculated based on K_i and K_i' were designated with the suffices of K_i and K_i' , respectively, as $\Delta G_{K_i}^{\circ}$, $\Delta G_{K_i'}^{\circ}$, and so forth.

3. Results

3.1. Purification of eight kinds of CGAs from DGCBE

Nine kinds of CGA derivatives were identified from a decaffeinated green coffee bean extract (Fig. 2). The extract was applied to a Sephadex LH-20 column, and collected as NF and AF (see "Materials and methods"). HPLC analysis showed that NF consisted of 3-CQA, 4-CQA, 5-CQA, 3FQA, 4-FQA, and 5-FQA, and that AF consisted of 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and non identified compounds (data not shown). AF and NF were applied to a Toyopearl HW-40F column, separately. Three peaks (PF-1, PF-2, and PF-3) were obtained from NF (Fig. 3A). PF-1 consisted of 3-CQA and 3-FQA; PF-2 of 4-CQA and 4-FQA; and PF-3 of 5-CQA and 5-FQA. On the other hand, 2 peaks (PF-4 and PF-5) were from AF (Fig. 3B). PF-4 consisted of 3,4-diCQA and non-identified components; and PF-5 of 3,5-diCQA and 4,5-diCQA. 3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were obtained by re-chromatography of above fractions from PF-1 to PF-5 using the same HPLC method.

(Fig. 2)

(Fig. 3)

3.2. Inhibition of PPA-I by CGAs and cinnamic acid derivatives

PPA-I-catalyzed hydrolysis of G₂-pNP was assayed in the presence and absence of inhibitors at pH 6.9 and 30 °C (Fig. 4). At 0.80 μM PPA-I and 8.0 mM G₂-pNP, the v_e value was determined to be 23.4 ± 0.7 nM s⁻¹. The relative activities of PPA-I decreased with increasing the concentrations of inhibitors and the respective IC₅₀ values were shown in Table 1. The IC₅₀ values of *m*-MCiA, *p*-MCiA, and CiA could not be determined because of their low solubility and low inhibitory effect.

(Fig. 4)

3.3. Inhibition model of CGAs and cinnamate derivatives against PPA-I

The lines obtained in the Hanes–Woolf plots for the PPA-I-catalyzed hydrolysis of G₂-pNP in the absence and presence of the respective concentrations of inhibitors except for 3,4-diCQA and 4,5-diCQA intersected each other on a point in the second quadrant (Fig. 5). However, the lines in the absence and presence of 40 μM 3,4-diCQA and 40 μM 4,5-diCQA intersected each other on a point in the third quadrant. These results suggest that the inhibition modes of 3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, DHCA, FA, IFA, *m*-CoA, *p*-CoA, *m*-MCiA, *p*-MCiA, and CiA against the PPA-I-catalyzed hydrolysis are mixed-type. The k_{cat} and K_m values of the hydrolysis were determined to be $(2.92 \pm 0.10) \times 10^{-2}$ s⁻¹ and 5.09 ± 0.35 mM, respectively. The apparent k_{cat} and K_m values in the presence of each inhibitor were shown in Table 1. The K_i and K_i' values of these inhibitors against PPA-I were calculated by Equation 2. The respective values were shown in Table 1.

(Fig. 5)

(Table 1)

The mixed-type inhibition suggests that there might be an inhibitor-binding site (or I-site) for CGAs and cinnamate derivatives examined above on the PPA-I surface in addition to the substrate-binding site (or S-site). For these inhibitors, the K_i' values are lower than their respective K_i values, indicating that they bind tighter to the I-site on the substrate-bound form of PPA-I than that on the free form. It should be noted that the K_m values in the presence of these inhibitors are lower than that in the absence. In other words, the substrate binding to the S-site decreases the binding affinity of these inhibitors to the I-site, whereas the inhibitor binding to the I-site increases the binding affinity of the substrate to the S-site. On the other hand, the K_i' values of 3,4-diCQA and 4,5-diCQA are higher than their K_i values, and the K_m values in the presence of 3,4-diCQA and 4,5-diCQA are higher than that in the absence, indicating that they bind tighter to the I-site on the free form of PPA-I than that on the substrate-bound form, and that the binding of them to the I-site decreases the binding affinity of the substrate to the S-site.

3.4. Temperature dependence of the inhibitor constants (K_i and K_i') of 5-FQA and 4,5-diCQA

The van't Hoff plots of K_i and K_i' values at pH 6.9 at the temperature of 20, 25, 30, 35, and 40 °C were examined (Fig. 6). From the slope of the plots, the ΔH° value for the binding of PPA-I with inhibitors (5-FQA, 3,5-diCQA and 4,5-diCQA) were determined. The ΔG° and ΔS° values at 25 °C were also calculated from Equations 3 and 4 (Table 2). These results indicated that the binding of PPA-I with 5-FQA, 3,5-diCQA or 4,5-diCQA was accompanied with a large ΔH° value, and thus the reaction was exothermic.

(Fig. 6)

(Table 2)

The interactions with 5-CQA, 5-FQA, 3,5-diCQA or 4,5-diCQA are exothermic and give a large increase in ΔH° , indicating that the interactions are enthalpy-driven. In the interaction between PPA and

inhibitors, the states of solvation might be changed accompanied with large changes in enthalpies and entropies to give enthalpy-entropy compensation, and thus with considerably small effect on ΔG° .

The ΔH° , ΔS° , and ΔG° values for the interactions of PPA-I with 5-CQA and 4,5-diCQA or 3,5-diCQA are compared (Table 2). The differences in ΔH°_{Ki} and $\Delta H^\circ_{Ki'}$ ($\Delta\Delta H^\circ_{Ki}$ and $\Delta\Delta H^\circ_{Ki'}$) between those of 5-CQA and 4,5-diCQA are 22.9 kJ mol⁻¹ and 6.0 kJ mol⁻¹, respectively, and the differences in $T\Delta S^\circ_{Ki}$ and $T\Delta S^\circ_{Ki'}$ ($\Delta T\Delta S^\circ_{Ki}$ and $\Delta T\Delta S^\circ_{Ki'}$) values are 29.0 kJ mol⁻¹ and 6.3 kJ mol⁻¹. The $\Delta\Delta G^\circ_{Ki}$ and $\Delta\Delta G^\circ_{Ki'}$ value between 5-CQA and 4,5-diCQA are -6.4 kJ mol⁻¹ and -0.4 kJ mol⁻¹. Introduction of the second CA moiety into 5-CQA to form 4,5-diCQA enhances the binding affinity of 5-CQA to E higher than that to ES. However, The $\Delta\Delta G^\circ_{Ki}$ and $\Delta\Delta G^\circ_{Ki'}$ value between 5-CQA and 3,5-diCQA are -1.9 kJ mol⁻¹ and -3.4 kJ mol⁻¹. Introduction of the second CA moiety into 5-CQA to form 3,5-diCQA enhances the binding affinity of 5-CQA to ES higher than that to E. These lines of evidence provide us valuable hints for development of α -amylase inhibitors useful for prevention of diabetes and obesity. Especially, introduction of the CA moiety in to QA is much more effective in PPA inhibition than introduction of the FA moiety, and introduction of two CA moieties is more effective than that of one moiety. This suggests that the inhibitory activity of QA seems to increase with increasing the number of CA moieties or the number of hydroxyl groups introduced. This tendency is observed also in the inhibition of MMP-7 by catechins (Oneda, Shiihara, & Inouye, 2003). The QA derivatives substituted with three or more CA moieties or with galloyl groups other than caffeoyl groups might improve inhibitory activities.

4. Discussion

4.1. Inhibitory activities of CGAs and cinnamate derivatives against PPA-I

We previously reported the inhibitory effects of 5-CQA and its components, CA and QA against PPA (PPA-I and PPA-II) with G₂-pNP as substrates at pH 6.9 and 30 °C (Narita & Inouye, 2009). The inhibitory activities of 5-CQA and CA are considerably high and follow the mixed-type inhibition mode.

1 However, the inhibitory activity of QA is much weaker and does not follow the conventional Michelis-
2 Menten-type inhibition mode. It should be noted that the inhibitory activities and behaviors of the
3 respective inhibitors against PPA isozymes are in good agreements, and their enzyme chemical
4 properties are almost the same except for the slight difference in pI values, suggesting that the inhibitory
5 activities of the inhibitors against PPA (the mixture of PPA-I and PPA-II) could be evaluated
6 sufficiently by the inhibition assay against only PPA-I. PPA-inhibitory activities of CGA derivatives
7 (other than 5-CQA) and the related compounds contained in green coffee beans are remained to be
8 revealed. In the present study, we examined the inhibitory activities of eight kinds CGAs; 3-CQA, 4-
9 CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA. The inhibitory activities of
10 nine kinds of CGAs and their components, CA, FA, and QA, against the PPA-I-catalyzed hydrolysis of
11 G_2 - p NP were in the order of 3,4-diCQA = 4,5-diCQA > 3,5-diCQA > 5-CQA > 4-CQA > 3-CQA > CA
12 > 5-FQA > 4-FQA > 3-FQA > FA >> QA by comparing the IC_{50} values (Table 1). The inhibitory
13 activity of diCQA is higher than that of CQA, which is higher than that of QA, suggesting that the
14 inhibitory activity of CGAs increases with increasing the number of CA sub-structure. The inhibitory
15 activities of CA derivatives are always higher than those of FA derivatives, suggesting that the
16 neighboring two hydroxyls on the catechol ring are effective in the inhibition. The substitution position
17 of the CA and FA sub-structures on QA in CQA and FQA is also significant in the inhibitory activity.
18 The substitute at 5-position is higher than that at 4-position, which is higher than that at 3-position.
19 However, the presence of the CA sub-structure at 4-position in diCQA gives higher inhibitory activity
20 than the absence. The inhibitory activities of 5-CQA and 3-CQA against rat intestine crude α -
21 glucosidase using a maltose were reported to be in the order of 5-CQA (IC_{50} = 2.99 mM) > 3-CQA (IC_{50}
22 = 3.12 mM) (Ishikawa et al., 2007), which agrees with that obtained here with PPA-I.

23 The inhibition potencies of FA, IFA, *m*-CoA, and *p*-CoA are almost the same (Table 1). It is
24 suggested that the methoxy group of benzene ring in FA and IFA does not much affect the inhibition.
25 The inhibitory activity of CA is 5 times higher than DHCA, suggesting that restriction of the free
26 rotation of the carbohydrate chain in CA by introducing an olefinic double bond contributes to enhance

1 the inhibitory activity. The similar inhibitory effect was observed in the inhibition of matrix
2 metalloproteinase 7 (MMP-7) which is closely related with the tumor invasion and metastasis by two
3 lignan species, (-)-secoisolariciresinol and (-)-matairesinol (Muta et al., 2004). The former is a
4 phenylpropanoid dimers connected with a single' bond, and the free rotation around this bond is
5 restricted in the latter by a butyrolactone ring. The inhibitory activity against MMP-7 is much improved
6 in the latter.

7 In the previous paper (Narita & Inouye, 2009), we proposed a hypothetical model for I-site based on
8 the IC_{50} values by mimicking the subsite structures demonstrated in the I-sites of proteinases (Inouye,
9 Lee, & Tonomura, 1996; Muta & Inouye, 2002). This model could be applicable to the inhibition of
10 PPA-I by CGAs. Namely, each of the diCQAs, CQAs, and FQAs binds to the I-site which is separated
11 into two subsites, CA and QA subsites. We have introduced a concept that the structure of CQA (and
12 FQA) is separated into CA (and FA) and QA sub-structures, which are linked with an ester bond to
13 form CQA (and FQA). The inhibitory potency of CA is enhanced 5, 3, and 2 times by combining with
14 QA to form 5-CQA, 4-CQA, and 3-CQA, respectively (Table 1). The potency of FA is also enhanced 5,
15 3, and 2 times by combining with QA to form 5-FQA, 4-FQA, and 3-FQA, respectively. This result
16 suggests that the CA and FA sub-structures bind to the CA subsite of the I-site. Substitution of the
17 methoxy group for the hydroxyl group in the catechol ring of CA reduces the binding affinity of CA to
18 the I-site by 10-20 times. This corresponds to the decrease of $4-8 \text{ kJ mol}^{-1}$ in ΔG^0 in the interaction
19 between CA and the enzyme, suggesting that the hydroxyl group in CA should be involved in forming a
20 single hydrogen bond with the CA subsite and this hydrogen bond could be failed by substituting the
21 hydroxyl with methoxy group in FA. The inhibitory activities of diCQAs are considerably higher than
22 those of CQAs, suggesting that there might be an additional binding subsite for the second CA sub-
23 structure. The Hanes–Woolf plots in the absence and presence of 3,5-diCQA intersected on a point in
24 the second quadrant (Fig. 5). Meanwhile, in the cases of 3,4-diCQA and 4,5-diCQA, the plots
25 intersected on a point in the third quadrant (Fig. 5), indicating that the inhibition mechanism of 3,5-
26 diCQA and that of the latter two diCQAs are different, while their inhibitory potencies are similar.

4.2. Inhibitor constants of nine kinds of CGAs and cinnamate derivatives against PPA-I

The inhibitor constants K_i and K_i' that correspond to the dissociation constants K_d of the EI complex into I plus E and that of the ESI complex into I plus ES complex, respectively, were determined (Table 1). 3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,5-diCQA CA, DHCA, FA, IFA, *m*-CoA, *p*-CoA, *m*-CiA, *p*-CiA, CiA show mixed-type inhibition with $K_i > K_i'$, suggesting that the inhibitors bind to the ES complex stronger than to the free enzyme E. In contrast, 3,4-diCQA and 4,5-diCQA show mixed-type inhibition with $K_i < K_i'$, suggesting that these inhibitors bind to E stronger than to ES.

The hydroxylation (*m*-CoA or *p*-CoA) at the meta or para position of the phenyl group of CiA reduces the K_i and K_i' values to 1/6 and 1/3 respectively, and the methoxylation (*m*-MCiA or *p*-MCiA) reduces only the K_i' values to 1/2 (Table 1). The hydroxylation at the meta or para position of the phenol ring of *p*-CoA or *m*-CoA respectively to form CA reduces the K_i and K_i' values to 1/7 and 1/11, and the methoxylation to form FA or IFA hardly changes the K_i and K_i' values. Furthermore, the hydroxylation at the meta or para position of the phenol ring of FA or IFA to form CA reduces the K_i and K_i' values to 1/8 and 1/17 respectively, and that at the meta or para position of the ring of 5-FQA to form 5-CQA reduces the K_i and K_i' values to 1/8 and 1/20 respectively. These results suggest that the PPA inhibition effect of the CA-related compounds is enhanced by modifying the hydroxyl group and is not much influenced by modifying the methoxy group in the cinnamate structure.

The K_i and K_i' values of 3-CQA and 3-FQA, 4-CQA and 4-FQA, and 5-CQA and 5-FQA are 1/2, 1/3, and 1/5 of the respective values of CA and FA (Table 1). This supports the hypothetical model in which CQAs and FQAs bind to the same I-site separated into two subsites (CA and QA subsites) accommodating the CA or FA sub-structure and QA sub-structure in CQAs or FQAs, respectively. The K_i and K_i' values of 3,4-diCQA are 0.01 and 0.02 mM, respectively, and those of 4,5-diCQA are 0.01 and 0.03 mM. However, those of 3,5-diCQA are 0.10 and 0.01 mM, respectively. The $\Delta\Delta H^\circ_{K_i}$ and $\Delta\Delta H^\circ_{K_i'}$ between those of 3,5-diCQA and 4,5-diCQA are 0.4 kJ mol⁻¹ and 21.8 kJ mol⁻¹, respectively,

1 and the differences in $\Delta T\Delta S^{\circ}_{Ki}$ and $\Delta T\Delta S^{\circ}_{Ki'}$ values are 4.7 kJ mol^{-1} and 16.7 kJ mol^{-1} . The $\Delta\Delta G^{\circ}_{Ki}$ and
2 $\Delta\Delta G^{\circ}_{Ki'}$ value between 3,5-diCQA and 4,5-diCQA are -4.3 kJ mol^{-1} and 3.0 kJ mol^{-1} . The difference of
3 the introduction position of the second CA moiety into 5-CQA form 3,5-diCQA or 4,5-diCQA affects
4 the binding affinity of 5-CQA to E and ES. These results suggest that the inhibition mechanism of 3,4-
5 diCQA and 4,5-diCQA could be similar, while 3,5-diCQA might follow different one. The fact that all
6 diCQAs show almost one-order stronger inhibitory potency than CQAs suggests the presence of an
7 additional subsite other than the CA and QA subsites. The PPA inhibition model of diCQAs is far
8 complicated to understand and needs further study. We are currently underway to examine the
9 interaction between the inhibitors and PPA-I and also to characterize the I-site by fluorescence and
10 surface-plasmon resonance analyses and chemical modification of PPA.

11

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Figure Captions

Fig. 1. Structures of chlorogenic acids in green coffee beans and cinnamate derivatives.

Fig. 2. A typical HPLC chromatogram of DGCBE at 325 nm. Peaks (retention times are given in parentheses, min): 1, 3-CQA (8.0); 2, 5-CQA (10.4); 3, 4-CQA (12.6); 4, 3-FQA (13.8); 5, 5-FQA (18.1); 6, 4-FQA (20.3); 7, 3,5-diCQA (29.7); 8, 3,4-diCQA (31.7); 9, 4,5-diCQA (33.0).

Fig. 3. (Panel A) A typical chromatogram of NF by Toyopearl HW-40F chromatography. Peaks 1, 2, and 3 contained 3-CQA and 3-FQA, 4-CQA and 4-FQA, and 5-CQA and 5-FQA, respectively. **(Panel B)** A typical chromatogram of AF by Toyopearl HW-40F chromatography. Peaks 4 and 5 contained 3,4-diCQA and some non-identified materials, and 3,5-diCQA, and 4,5-diCQA, respectively. The detection was done with absorption at 325 nm

Fig. 4. Inhibitory effects of CGAs and cinnamate derivatives against the PPA-I-catalyzed hydrolysis of G_2 - p NP. The inhibition assay was performed in buffer B containing 25 mM NaCl at pH 6.9 and 30 °C. **(Panel A)** The symbols: 3-CQA (open circles), 4-CQA (open triangles), 5-CQA (open squares), 3-FQA (solid circles), 4-FQA (solid triangles), 5-FQA (solid squares), 3,4-diCQA (open diamonds), 3,5-diCQA (solid diamonds), and 4,5-diCQA (inverted triangles). **(Panel B)** The symbols: CA (open circles), FA (open triangles), p -CoA (open squares), DHCA (solid circles), IFA (solid triangles), m -CoA (solid squares), p -MCiA (open diamonds), m -MCiA (solid diamonds), and CiA (inverted triangles). The initial concentrations of PPA-I and G_2 - p NP were 0.80 μ M and 8.0 mM, respectively. As for other conditions, see the "Materials and methods". Each point represents the means of triplicate experiments.

Fig. 5. Hanes-Woolf plots for the PPA-I-catalyzed hydrolysis of G₂-pNP. PPA-I activity was measured in the presence of 3-, 4-, and 5-CQA (Panel A), 3-, 4-, and 5-FQA (Panel B), 3,4-, 3,5-, and 4,5-diCQA (Panel C), and cinnamate derivatives (Panel D) under the same conditions as in Figure 4. **(Panel A)** The initial concentrations of 3-CQA, 4-CQA, and 5-CQA: 0 M (open circles), 0.3 mM (solid triangles), 0.1 mM (solid squares), and 0.2 mM (solid diamonds). **(Panel B)** Those of 3-FQA, 4-FQA, and 5-FQA: 0 M (open circles), 3.0 mM (solid triangles), 2.0 mM (solid squares), and 0.8 mM (solid diamonds). **(Panel C)** Those of 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA: 0 M (open circles), 40 μM (solid triangles), 40 μM (solid squares), 40 μM (solid diamonds). **(Panel D)** Those of FA, IFA, DHCA, CiA, *m*-CoA, *p*-CoA, *m*-MCiA, and *p*-MCiA: 0 M (open circles), 5.0 M (open triangles), 5.0 mM (open squares), 2.0 mM (open diamonds), 6.0 mM (inverted triangles), 5.0 mM (solid circles), 5.0 mM (solid triangles), 4.0 mM (solid squares), and 4.0 mM (solid diamonds). Each point represents the means of triplicate experiments.

Fig. 6. van't Hoff plots of K_i and K_i' evaluated at pH 6.9 in the range of 20 – 40 °C. Open symbols: K_i values of the complexes of PPA-I with 5-FQA (open circles), 4,5-diCQA (open triangles), and 3,5-diCQA (open squares). Closed symbols: K_i' values of the complexes of PPA-I with 5-FQA (solid circles), 4,5-diCQA (solid triangles), and 3,5-diCQA (solid squares). Each point represents the means of triplicate experiments.

Table 1

Inhibitor constants, K_m , k_{cat} , and IC_{50} of nine chlorogenic acids from green coffee beans and cinnamate derivatives^a

Inhibitor	IC_{50} (mM)	K_i (mM)	K_i' (mM)	K_m (mM)	k_{cat} ($\times 10^{-2} s^{-1}$)
3-CQA	0.23 ± 0.03	0.61 ± 0.16	0.13 ± 0.01	2.37 ± 0.35	1.63 ± 0.04
4-CQA	0.12 ± 0.01	0.24 ± 0.03	0.10 ± 0.01	3.77 ± 0.38	2.73 ± 0.13
5-CQA	0.08 ± 0.02^b	0.23 ± 0.02^b	0.05 ± 0.01^b	3.04 ± 0.38^b	1.52 ± 0.21^b
3-FQA	2.55 ± 0.12	6.14 ± 0.49	1.74 ± 0.05	2.34 ± 0.03	1.85 ± 0.10
4-FQA	2.02 ± 0.09	4.34 ± 0.57	1.40 ± 0.39	2.97 ± 0.09	2.46 ± 0.02
5-FQA	1.09 ± 0.03	1.95 ± 0.11	0.99 ± 0.03	4.51 ± 0.19	3.16 ± 0.03
3,4-diCQA	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	7.70 ± 0.29	1.87 ± 0.13
3,5-diCQA	0.03 ± 0.00	0.10 ± 0.00	0.01 ± 0.00	1.18 ± 0.18	0.89 ± 0.02
4,5-diCQA	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	9.02 ± 0.91	2.42 ± 0.15
CA	0.40 ± 0.03^b	1.12 ± 0.14^b	0.27 ± 0.04^b	3.04 ± 0.38^b	2.31 ± 0.21^b
DHCA	1.94 ± 0.10	10.21 ± 1.91	1.61 ± 0.02	3.11 ± 0.31	2.55 ± 0.06
FA	5.45 ± 0.12	11.17 ± 0.91	4.87 ± 0.37	3.15 ± 0.08	2.76 ± 0.30
IFA	4.27 ± 0.17	7.16 ± 0.58	4.11 ± 0.08	3.42 ± 0.20	2.50 ± 0.04
<i>m</i> -CoA	4.51 ± 0.20	8.12 ± 1.12	3.31 ± 0.03	3.52 ± 0.20	2.22 ± 0.03
<i>p</i> -CoA	4.86 ± 0.27	7.82 ± 0.25	3.88 ± 0.14	3.89 ± 0.30	2.43 ± 0.08
<i>m</i> -MCiA	$> 4.5^c$	39.46 ± 3.29	4.96 ± 0.46	3.30 ± 0.10	3.05 ± 0.05
<i>p</i> -MCiA	$> 4.5^c$	45.83 ± 7.41	3.63 ± 0.32	2.81 ± 0.10	2.63 ± 0.05
CiA	$> 6.0^c$	45.99 ± 10.08	8.69 ± 0.84	3.64 ± 0.24	3.26 ± 0.06
QA	26.5 ± 1.8^b	NA ^d	NA ^d	NA ^d	NA ^d

^a A mean of triplicate analysis \pm SD.

^b Narita & Inouye (2009).

^c Not determined correctly because of the low solubility of the inhibitor.

^d NA, not analyzed.

1

Table 2

Thermodynamic binding parameters of 5-CQA, 5-FQA, 3,5-diCQA, and 4,5-diCQA with PPA-I

(parameter unit: kJ mol⁻¹)^a

inhibitor	ΔG°_{Ki}	$\Delta G^{\circ}_{Ki'}$	ΔH°_{Ki}	$\Delta H^{\circ}_{Ki'}$	$T\Delta S^{\circ}_{Ki}$	$T\Delta S^{\circ}_{Ki'}$
5-CQA	-21.7 ± 1.7^b	-25.5 ± 1.9^b	-70.3 ± 4.3^b	-59.0 ± 3.6^b	-48.5 ± 3.9^b	-33.4 ± 3.7^b
5-FQA	-16.2 ± 0.3	-17.3 ± 0.2	-40.5 ± 2.4	-36.9 ± 2.1	-24.3 ± 2.5	-19.6 ± 2.2
3,5-diCQA	-23.6 ± 0.7	-28.9 ± 0.3	-47.8 ± 6.4	-74.8 ± 2.2	-24.2 ± 5.8	-43.8 ± 2.1
4,5-diCQA	-27.9 ± 0.3	-25.9 ± 0.8	-47.4 ± 5.1	-53.0 ± 1.7	-19.5 ± 5.3	-27.1 ± 0.8

2 ^a A mean of triplicate analysis \pm SD. ΔG° and $T\Delta S^{\circ}$ values were given at 25 °C.3 ^b Narita & Inouye (2009).

4

5

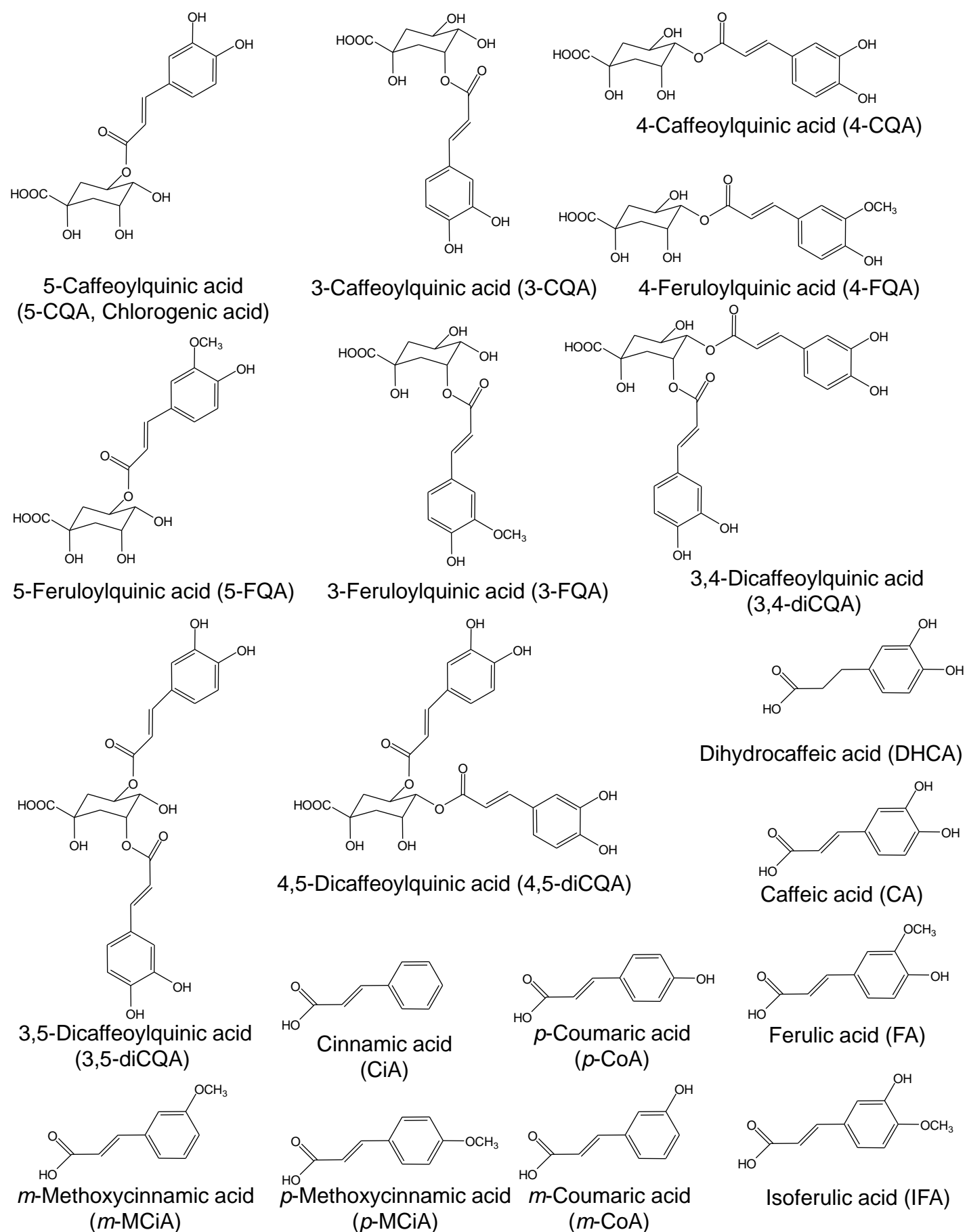


Fig. 1

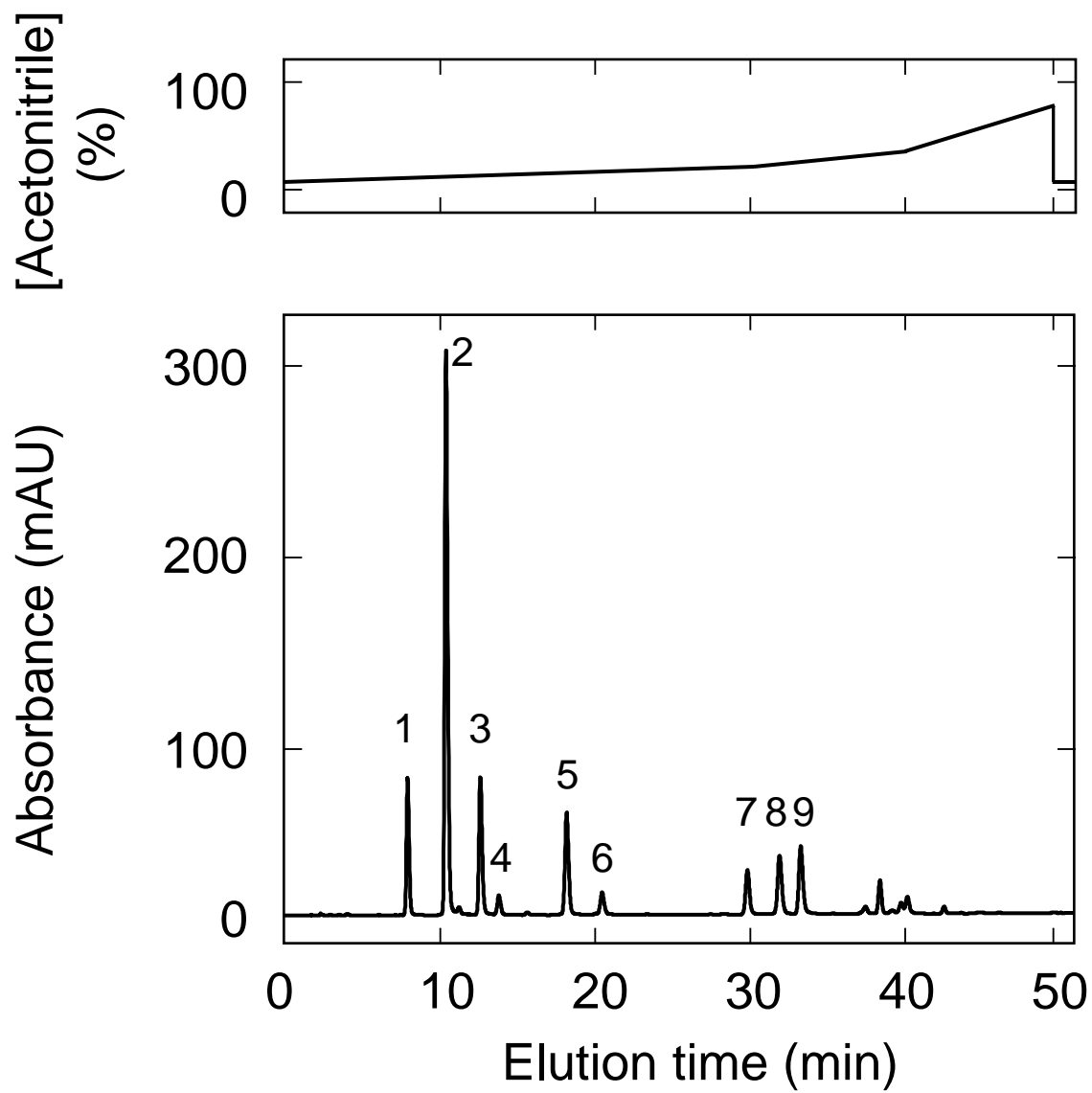


Fig. 2

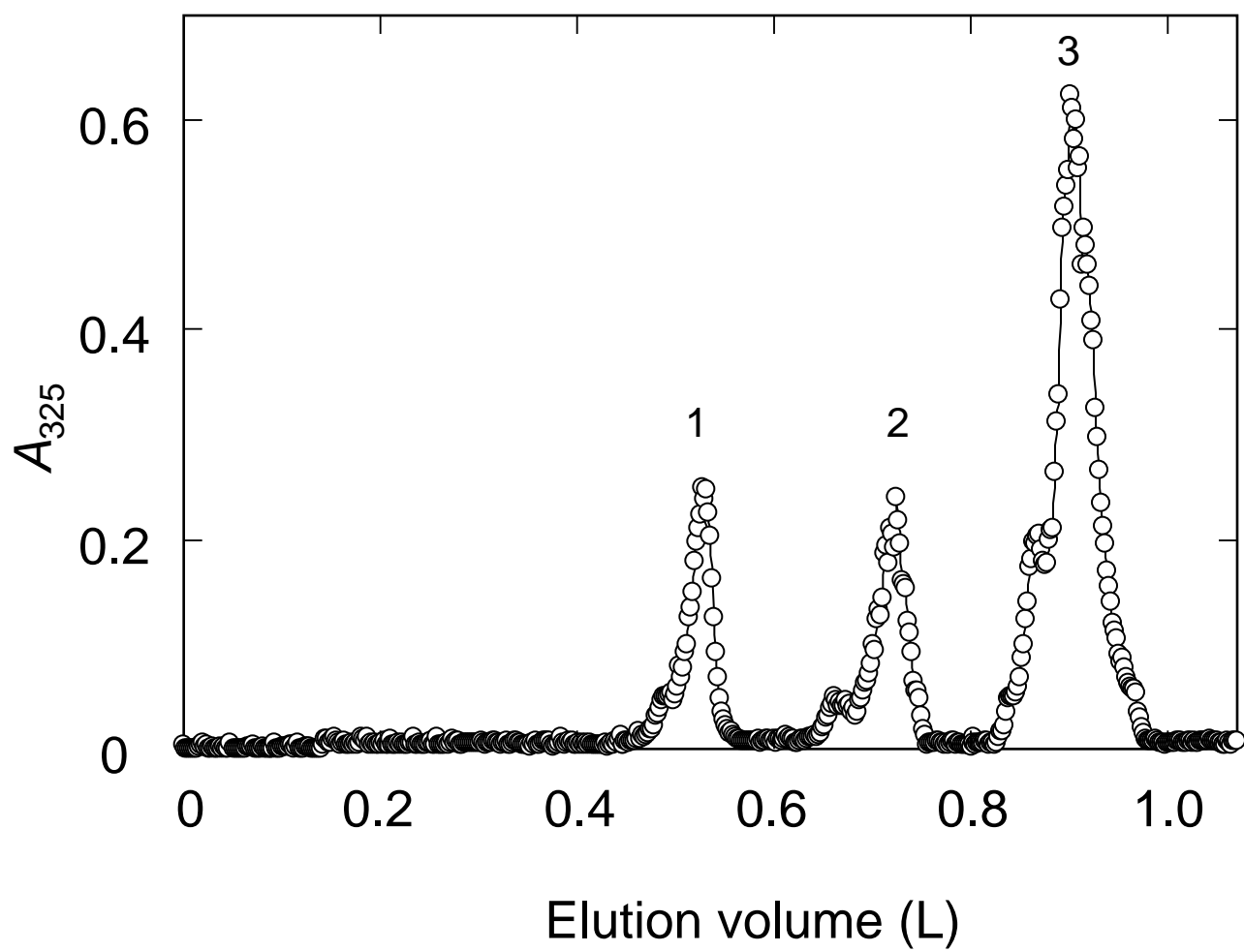


Fig. 3A

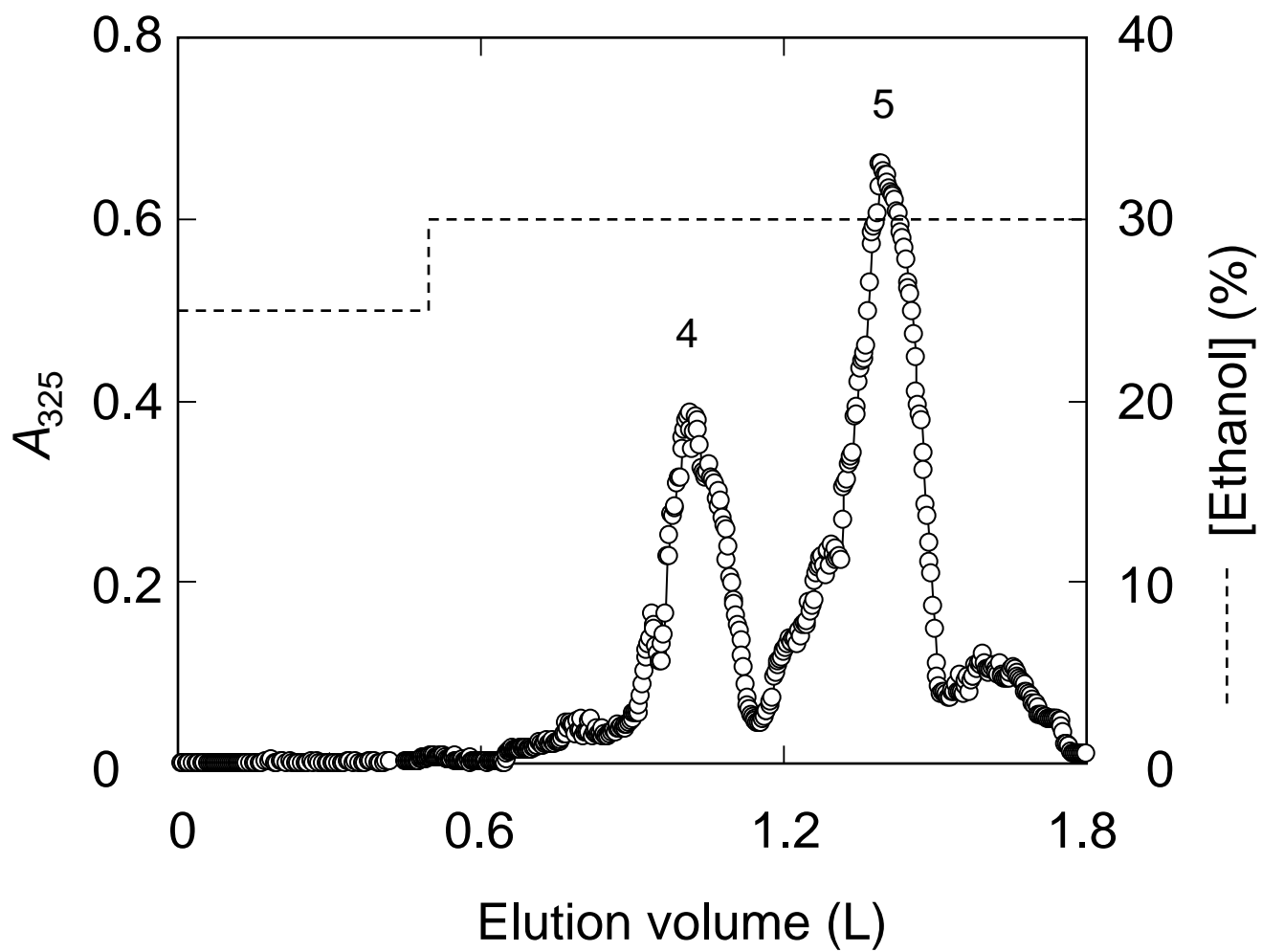


Fig. 3B

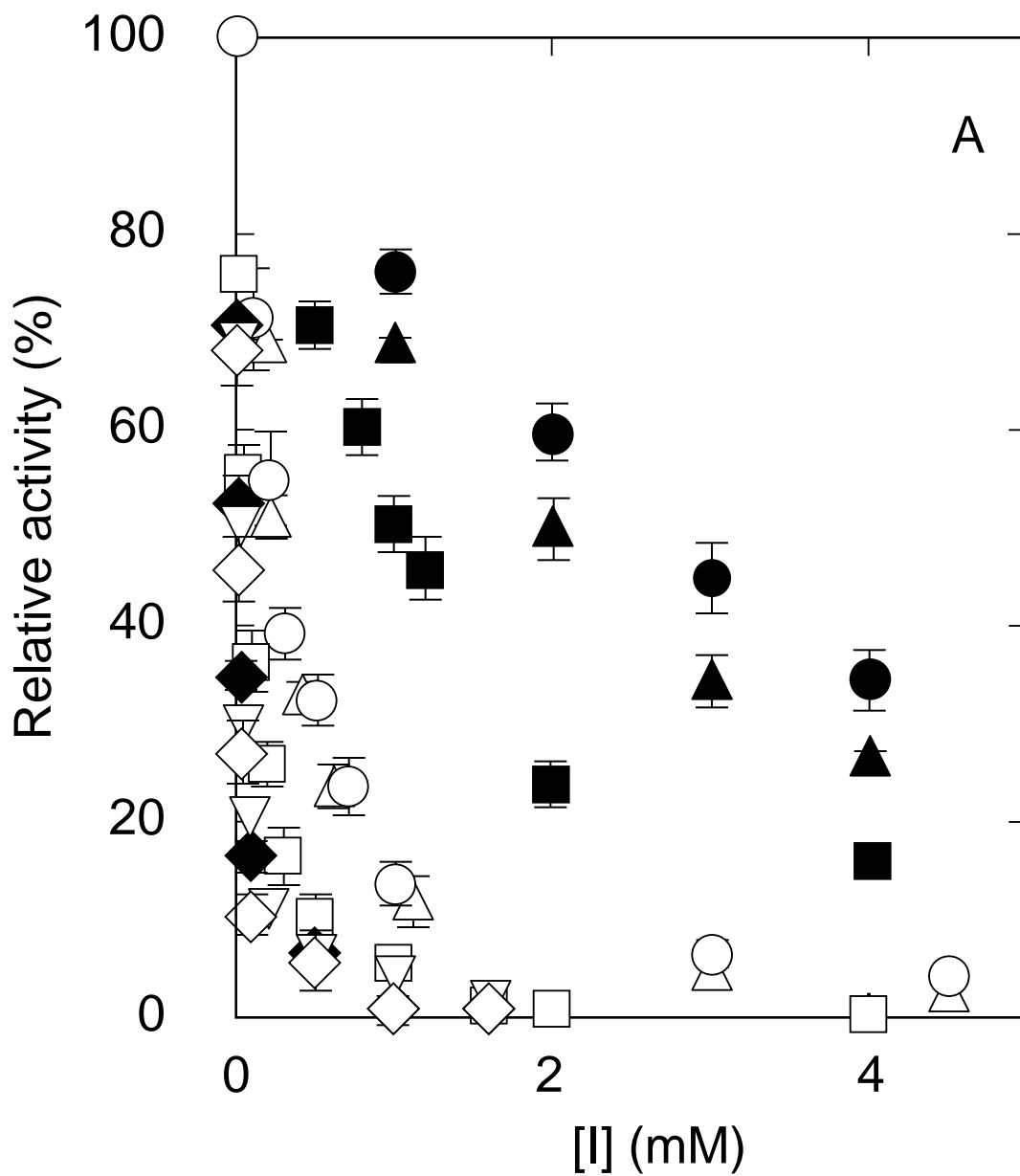


Fig. 4A

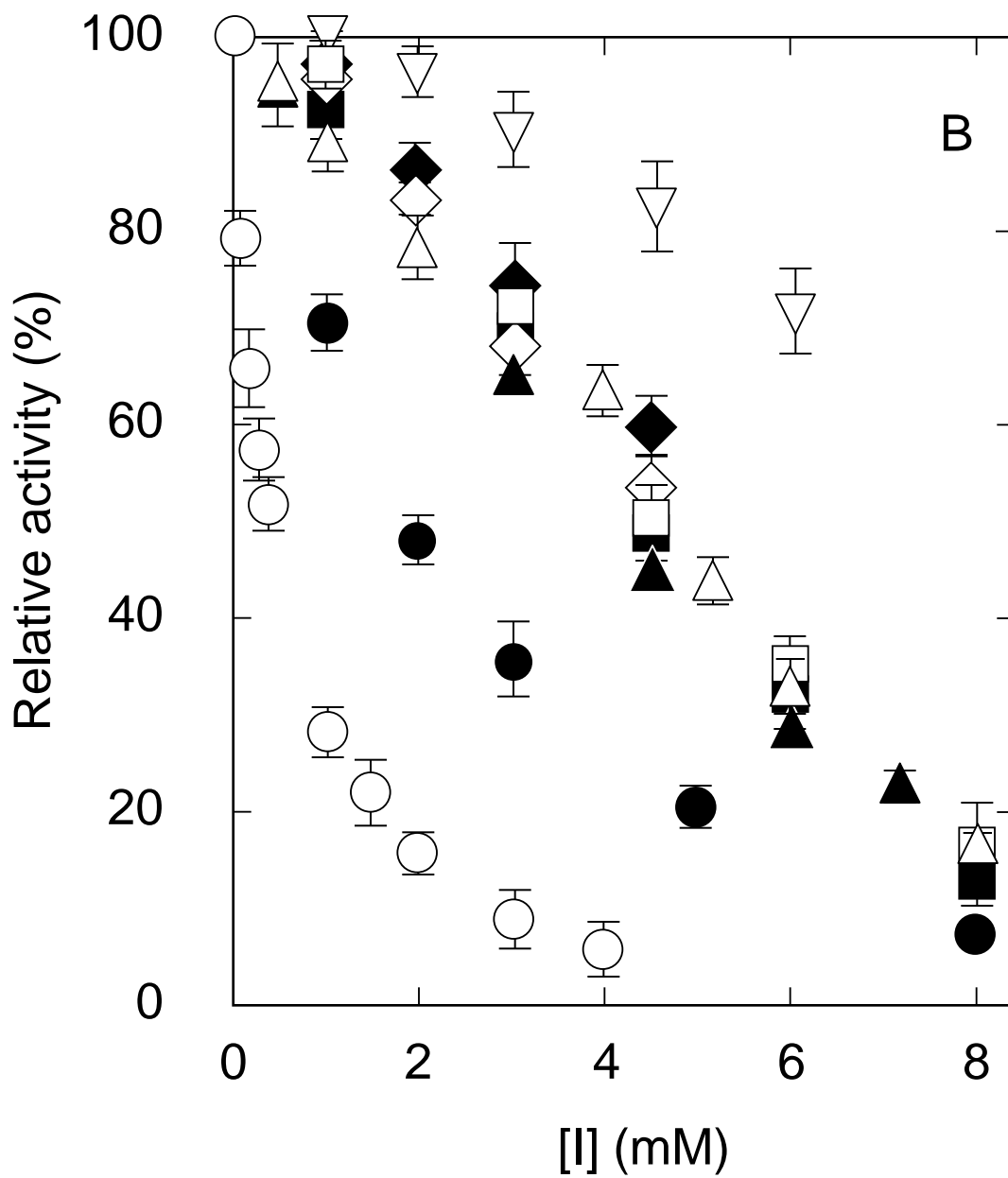


Fig. 4B

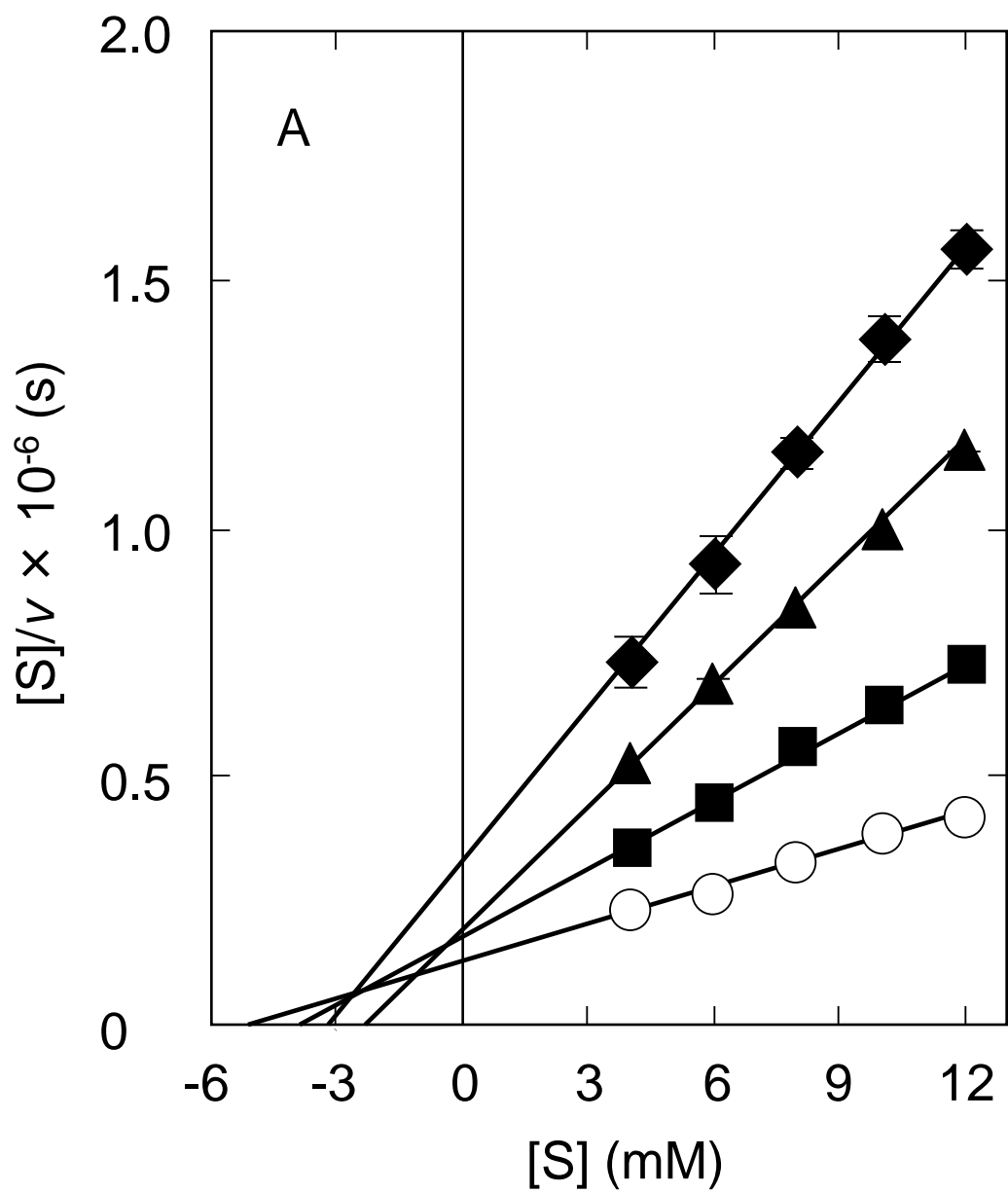


Fig. 5A

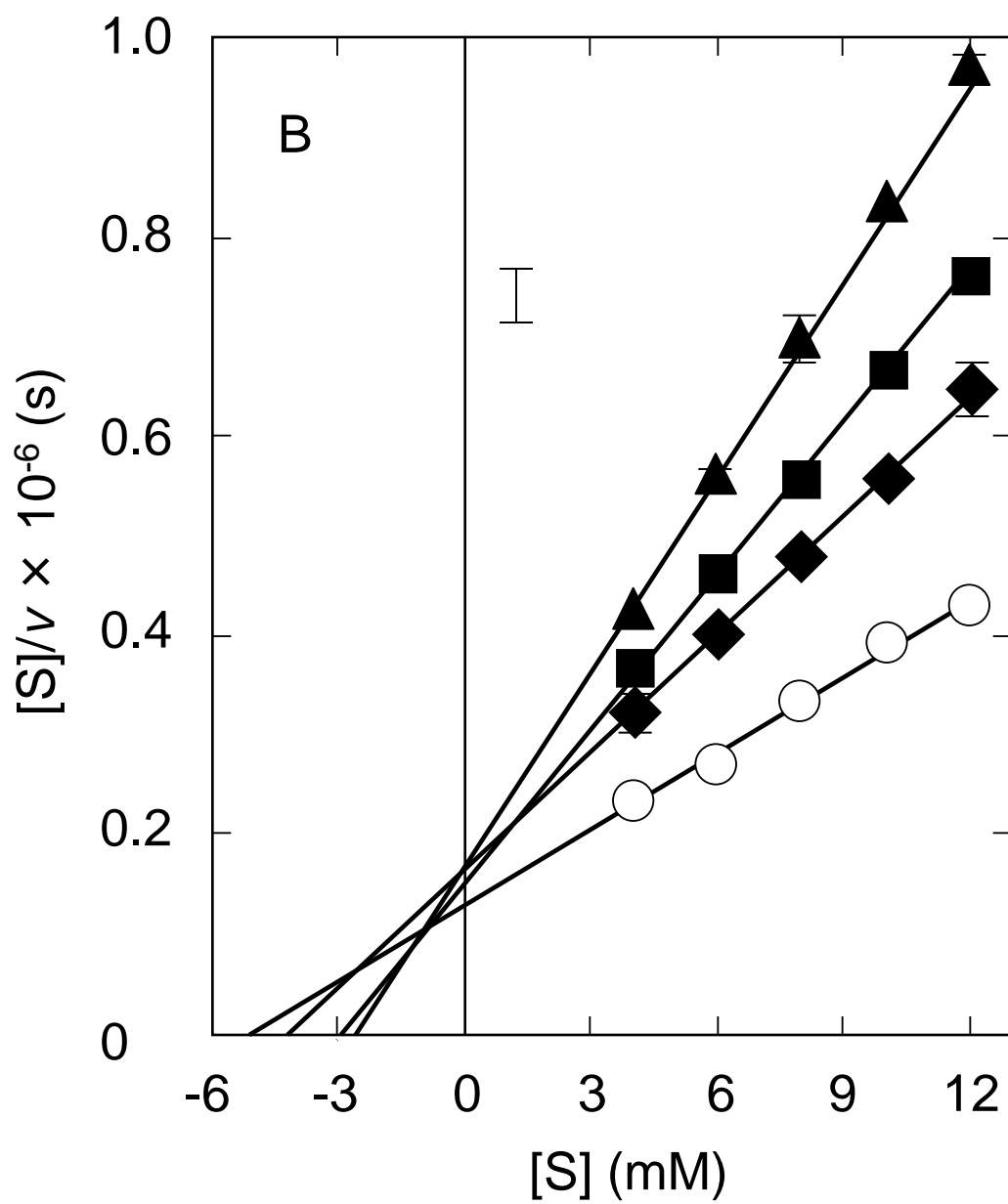


Fig. 5B

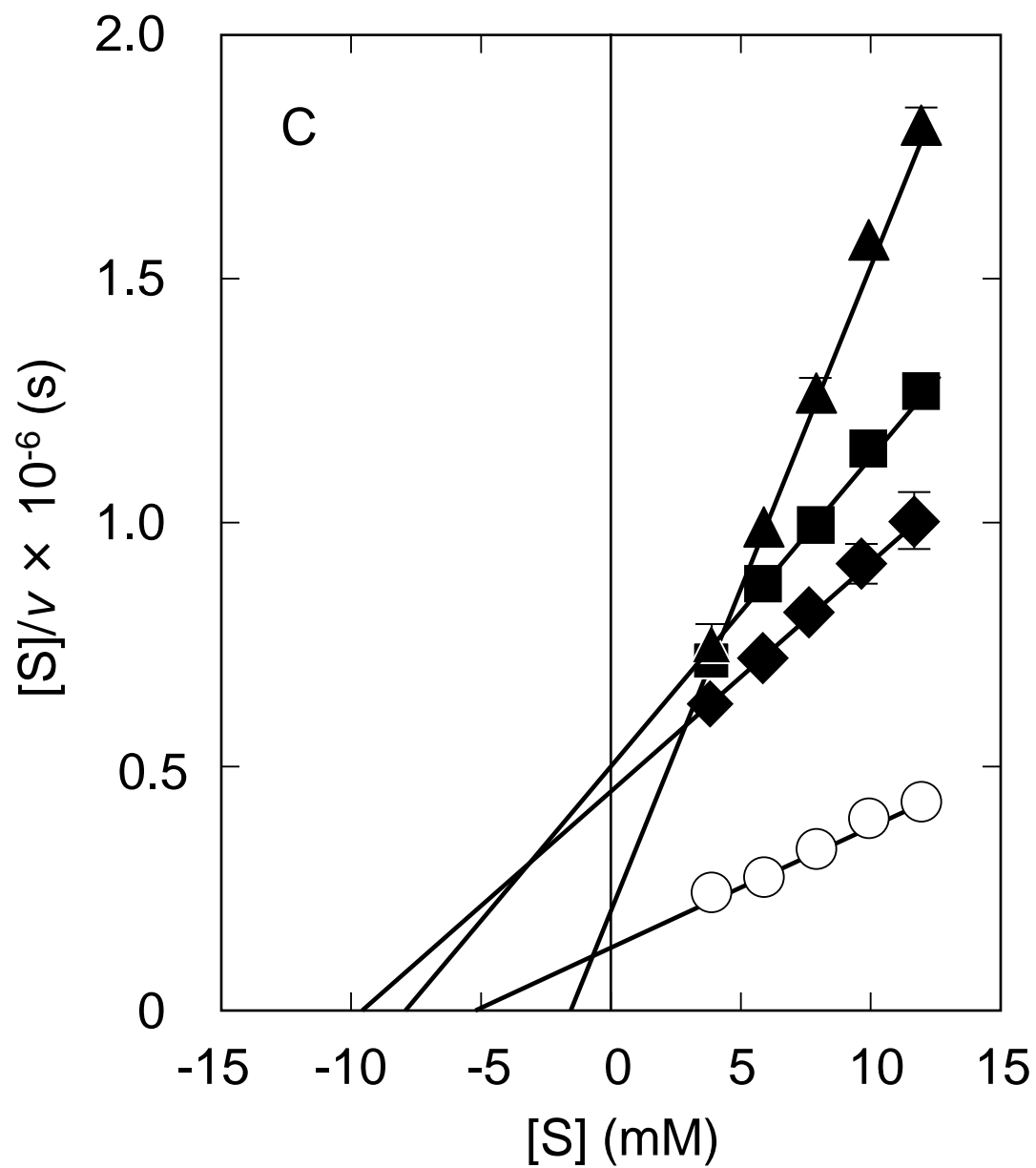


Fig. 5C

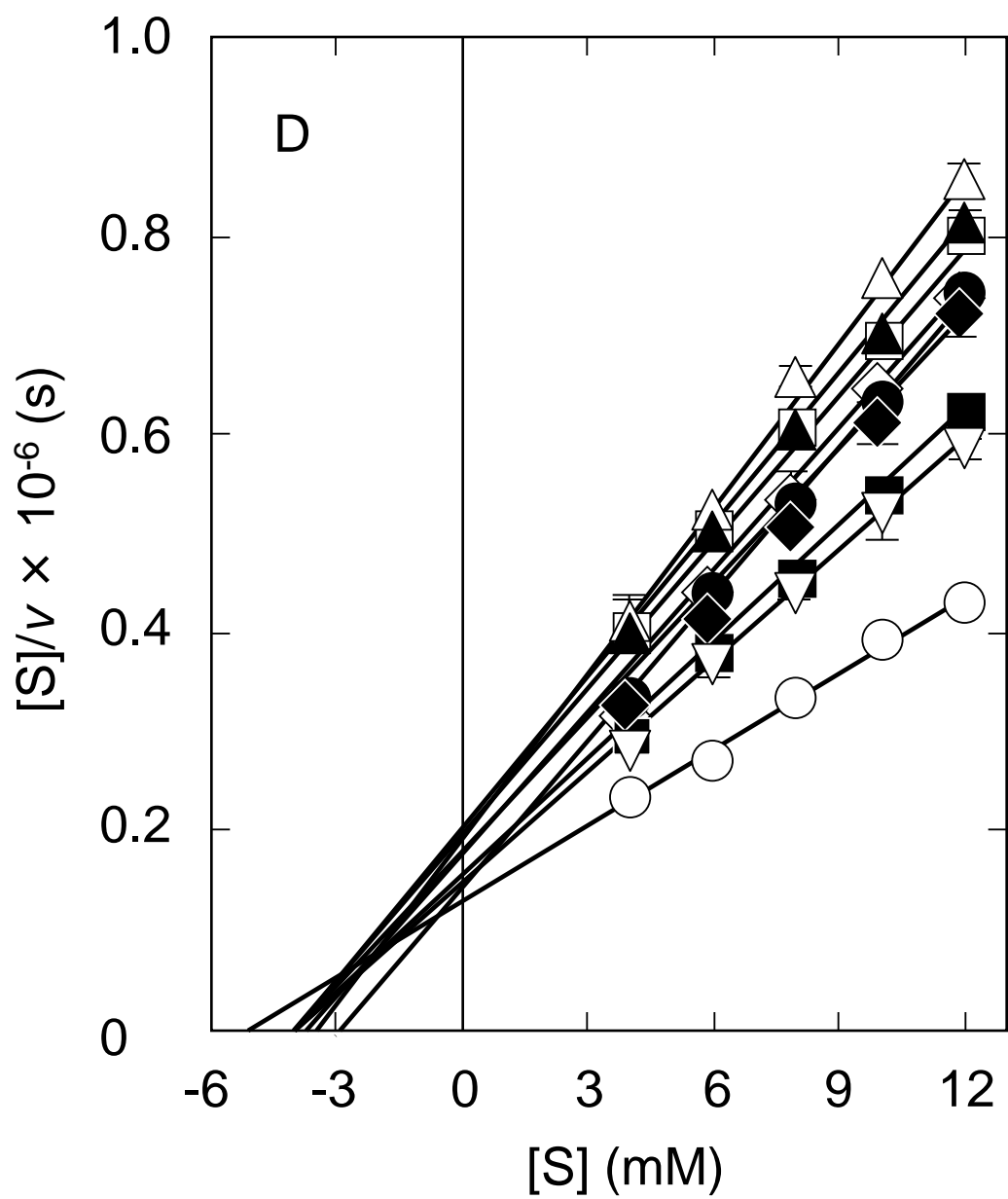


Fig. 5D

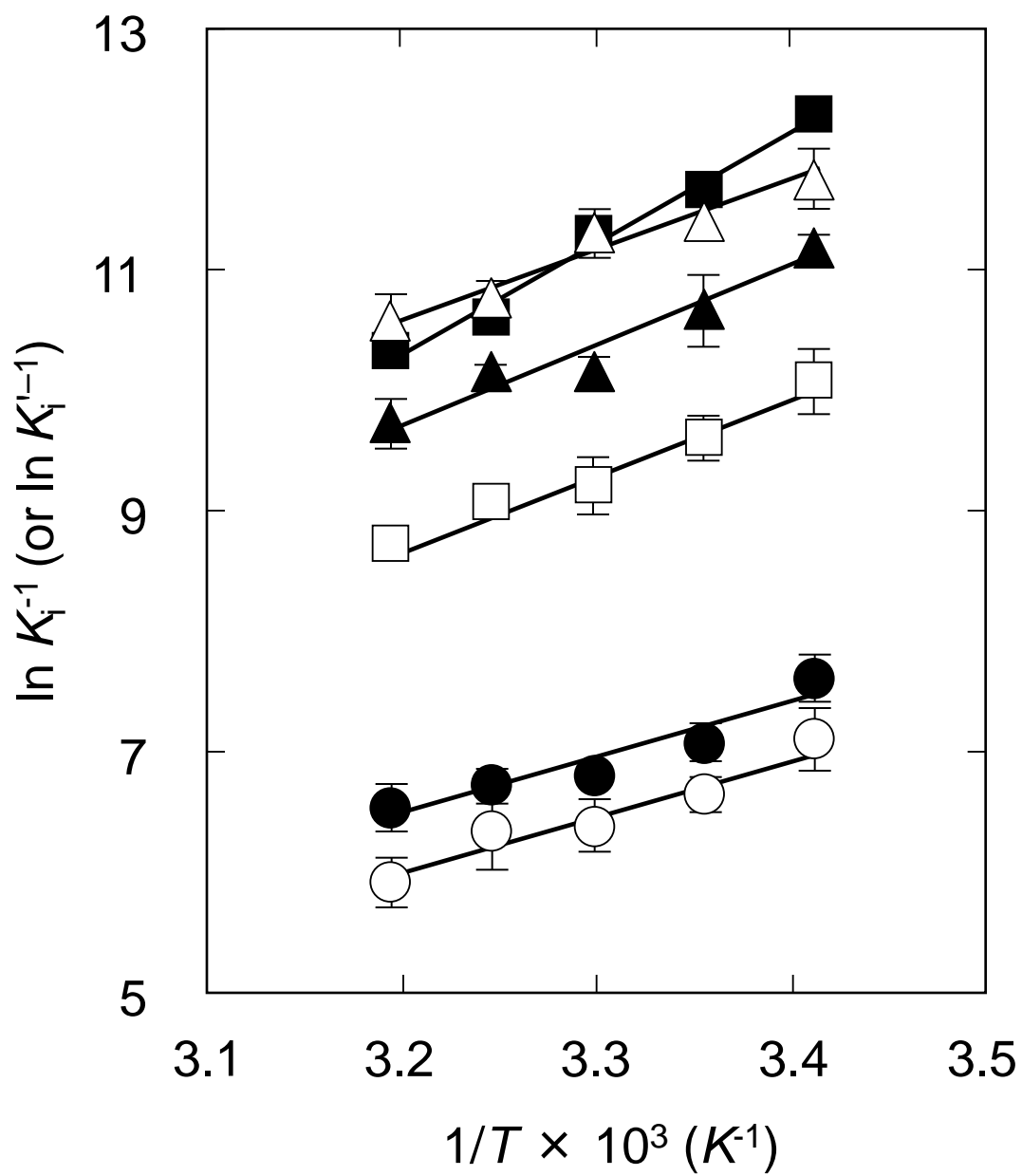


Fig. 6